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14. ABSTRACT Our previous studies indicated that li- vaccines are more efficient in CD4+ T-cell activation than li+ cells, and that mouse MHC II vaccines caused regression of established tumors in mice. In vitro studies with human MHC II vaccines demonstrated that the li- vaccines activated a population of CD4+ T-cells that is distinct from the population activated by li+ cells. This observation is consistent with our hypothesis that the absence of li results in the presentation of novel MHC II peptides. We have now identified peptides of four vaccines: MCF10/DR7/CD80, MCF10/DR7/CD80/li, MCF10/CIITA/CD80, and MCF10/CIITA/CD80/li siRNA. For every vaccine cell line two affinity purifications were performed, and for every affinity purification two LC-MS/MS runs were conducted. Peptides present in both affinity purifications and with Peptide Prophet probability scores above 0.05 were further analyzed. One hundred sixteen peptides were identified for MCF10/DR7/CD80 and 228 peptides for MCF10/DR7/CD80/li; 52 peptides were present in both cell lines (Fig 1A). One hundred eight peptides were identified for MCF10/CIITA/CD80 and 28 peptides for MCF10/CIITA/CD80/li siRNA cells, with 6 peptides common to both cell lines (Fig 1B). These findings are consistent with our hypothesis that li+ and li- MHC II vaccines present distinct peptide repertoires. Seven peptides were chosen to be best binders by Artificial Neural Net MHC II peptide prediction by our bioinformatician collaborator Dr. Michael O'Neill. All of these seven peptides have been shown to be immunogenic.					
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Introduction

Our goal is to create an efficient vaccine for the treatment of metastatic breast cancer and for prophylactic treatment of individuals at high-risk of developing breast cancer. Immune suppression induced by T-regs and MDSC is a major obstacle in immunotherapy [1, 2]. Tolerance to self antigens is another major impediment [3]. To overcome these problems, we are identifying novel tumor peptides to which the host has not previously been exposed and whose immunogenicity is not affected by immune suppression. We are focusing on identifying novel MHC class II-restricted peptides because $CD4^+$ T lymphocytes are essential for the optimal activation of cytotoxic $CD8^+$ T cells and for the generation of long-term immune memory, which is necessary for protection against latent metastatic disease [4, 5]. MHC II-restricted antigens are normally presented by professional antigen-presenting cells (APC), which endocytose exogenous proteins and degrade them to peptides in endosomal compartments. These peptides are bound by newly synthesized MHC II molecules which are directed to the endosomal compartments by the Invariant Chain (Ii), a chaperone that blocks the MHC II peptide binding groove so that peptides derived from *endogenously* synthesized proteins cannot prematurely occupy the peptide binding site [6]. In contrast to professional APC, our “MHC II” breast cancer vaccines do not contain Ii chain. MHC II vaccines are generated by transducing MHC I⁺Ii⁻ tumor cells with CD80 costimulatory genes and MHC II HLA-DR molecules that are syngeneic to the prospective recipient. *We hypothesize that in the absence of Ii the vaccine cells are non-traditional APC and their MHC II molecules present an antigen repertoire of endogenous origin which is distinct from the MHC II peptide repertoire presented by professional Ii⁺ APC.* Since breast cancer patients have not previously been exposed to these novel peptides, they will not be tolerized to them and the peptides should be highly immunogenic. In vivo experiments in mice demonstrated that Ii⁻ MHC II vaccines cause regression of established tumors, and human mammary carcinoma vaccines stimulated a much stronger $CD4^+$ T-cell response in vitro than Ii⁺ APC [7]. We have also demonstrated that human lung cancer MHC II vaccines activate $CD4^+$ T-cells despite the presence of immune suppression [8]. Therefore, the MHC II vaccines are an ideal source of novel immunogenic MHC II-restricted peptides. Using an Ii⁻ MHC II breast cancer vaccine prepared by transducing MCF10 mammary carcinoma cells with the MHC II transactivator, CD80, and an siRNA for Ii, I have isolated and identified candidate peptides. I am currently identifying peptides from Ii⁺ cells (MCF10/CIITA/CD80) and will compare them to Ii⁻ MHC II vaccine-derived peptides. I will then select peptides unique to the Ii⁻ MHC II vaccine and test them in vitro for their ability to activate $CD4^+$ T cells from breast cancer patients.

The following aims will use the MCF10/CIITA/CD80 vaccines and a single allele vaccine (MCF10/DR7/CD80).

Aim 1. Using mass spectrometry, identify peptides uniquely presented by Ii⁻ MCF10 MHC II vaccines and test the hypothesis that the absence of Ii facilitates the presentation of novel MHC II tumor peptides.

Aim 2. Determine if the peptides identified in Aim 1 activate type 1 tumor-reactive $CD4^+$ T-cells from breast cancer patients and if these peptides activate $CD4^+$ T-cells in the presence of immune suppression.

Aim 3. Determine if $CD4^+$ T-cells activated with the candidate peptides facilitate a more robust $CD8^+$ T-cell response by breast cancer patients.

Completion of these aims will provide “proof-of-principle” that the absence of Ii facilitates presentation of novel MHC II peptides, and will identify HLA-DR-restricted breast cancer peptides for therapeutic vaccines for patients with metastatic breast cancer.

Body

Specific Aim 1 – Completed

Our previous studies indicated that Ii^- vaccines are more efficient in $CD4^+$ T-cell activation than Ii^+ cells, and that mouse MHC II vaccines caused regression of established tumors in mice [5]. In vitro studies with human MHC II vaccines demonstrated that the Ii^- vaccines activated a population of $CD4^+$ T-cells that is distinct from the population activated by Ii^+ cells [7]. This observation is consistent with our hypothesis that the absence of Ii results in the presentation of novel MHC II peptides. We have now identified peptides of four vaccines: MCF10/DR7/CD80, MCF10/DR7/CD80/ Ii , MCF10/CIITA/CD80, and MCF10/CIITA/CD80/ Ii siRNA. For every vaccine cell line two affinity purifications were performed, and for every affinity purification two LC-MS/MS runs were conducted. Peptides present in both affinity purifications and with Peptide Prophet probability scores above 0.05 were further analyzed. One hundred sixteen peptides were identified for MCF10/DR7/CD80 and 228 peptides for MCF10/DR7/CD80/ Ii ; 52 peptides were present in both cell lines (Fig 1A). One hundred eight peptides were identified for MCF10/CIITA/CD80 and 28 peptides for MCF10/CIITA/CD80/ Ii siRNA cells, with 6 peptides common to both cell lines (Fig 1B). These findings are consistent with our hypothesis that Ii^+ and Ii^- MHC II vaccines present distinct peptide repertoires. Seven peptides were chosen to be best binders by Artificial Neural Net MHC II peptide prediction by Dr. Michael O'Neill.

Specific Aim 2 – in progress

Seven peptides identified in aim 1 (table 1) are already synthesized in the UMB biopolymer facility and T cell activation are assessed as previously described [7]. Briefly, peripheral blood mononuclear cells (PBMC) from HLA-DR7⁺ healthy donors are used for $CD4^+$ T-cell activation experiments. To determine if the peptides identified in Objective 1 are physiologically relevant PBMC are primed by pulsing them with one of the seven peptides and incubated at 37⁰. In parallel the same PBMC sample is primed by pulsing with Her2 p776 peptide as a positive control. Her2 p776 peptide is a well-studied DR7-restricted MHC II antigen, that is highly immunogenic and breast cancer specific [9]. After 6 days of incubation, cells are harvested, and expanded in medium containing 20 ng/ml of IL-15 (24 well plates at 1x10⁶ cells/2ml per well) for an additional 7 days. Expanded $CD4^+$ T cells are then rested without any cytokines for 24 hours. After resting $CD4^+$ T cells are boosted for 48 hours with irradiated MCF10/DR7/CD80, MCF10/CIITA/CD80, MCF10/CIITA/CD80/ Ii siRNA, MCF10 parental cells or non-malignant MCF10A cells, and the resulting supernatants analyzed for IFN γ secretion by ELISA. If the candidate peptides are relevant tumor peptides, then the primed $CD4^+$ T cells will react (produce IFN γ) when boosted with the corresponding MCF10 vaccine cells, but not when boosted with Ii^+ MCF10 cells or with parental MCF10 or non-malignant MCF10A cells. I have tested all 7 peptide candidates. Some of the peptides I have tested 2-3 times. Two individual examples of T-cell activation data are shown in Figure 2. As a result of 12 successful T-cell activation experiments we can conclude that all seven candidate peptides tested so far seem to be immunogenic and capable of eliciting T-cell response (Table 2). All of the 7 peptides are relevant tumor peptides because the highest IFN γ is secreted when T-cells are boosted with the corresponding MCF10 vaccine cells, but not when boosted with Ii^+ MCF10 cells or with parental MCF10 or non-malignant MCF10A cells. We acquired stage III breast cancer patient's PBMC and were able to conduct T-cell activation assay with them. As a result I was able to show that peptide 48 is highly efficient in activating breast cancer patient's PBMC (Fig 3). We are currently in process of preparing a manuscript for publication of this work.

Key Research Accomplishments

Training plan

Task 1. Meet with my dissertation committee once a year to review my progress in the project. (Completed)

Task 2. Meet with my mentor weekly to discuss ongoing experiments and come up with and implement new experiments. Discuss my mass spectrometry data at least monthly with Dr. Fenselau; more frequently if I encounter technical problems. Update Dr. Disis periodically on candidate peptides for potential clinical trials. (Completed)

Task 3. Complete all bench laboratory research necessary to fulfill the objectives of the research proposal. (in progress)

Task 4. Complete coursework required by Molecular and Cell Biology (MOCB) Ph.D. program. (Completed)

Task 5. Oral examination on the background of my research area, and presentation and defense of my research proposal at a comprehensive “preliminary/qualifying” exam, as required by the MOCB Ph.D. program. (Completed)

Task 6: Present my research findings at one or two national conferences per year. I have presented my findings at 3 national conferences in 2011 (see reportable outcomes section)

Task 7. Write up experimental results in a timely fashion and submit them for publication to peer-reviewed journals. (I have one manuscript that has been accepted for publication and published in March 2011; See reportable outcomes section). I am currently working on my second manuscript.

Task 8. Collaborate with other students and investigators as necessary to complete my project. (on-going)

Task 9. Participate in discussions and present my work at weekly journal clubs, seminars, lab meetings, and meetings with outside speakers. (on-going)

Task 11. Serve as a teaching assistant for two semesters. (Completed).

Milestones and Deliverables:

1. Completion of my preliminary/qualifying exam). (November 2008)
2. Completed all coursework to fulfill the MOCB Ph.D. program course requirements.
3. Completed 2 semesters as a teaching assistant.
4. Completed 4 different oral presentations at 4 prestigious national conferences. (See reportable outcomes)
5. My first peer-reviewed publication was published in Molecular and Cellular Proteomics journal in March 2011. (See reportable outcomes)
6. I was also a co-author on another manuscript that was published in Blood journal in May 2011. (See reportable outcomes)

Research Plan

Task 1. Identify peptides uniquely presented by Ii^- breast cancer vaccines and test the hypothesis that the absence of Ii facilitates the presentation of novel MHC II-restricted tumor peptides (completed). See fig. 1

Task 1a. Isolate MHC II-bound peptides from Ii^- and Ii^+ MCF10 cells (completed). See fig. 1

Task 1b. Using mass spectrometry analysis, compare the peptide repertoires produced by Ii^+ vs. Ii^- APC and identify candidate peptides using SEQUEST (completed). See fig. 2

Outcomes/Products/Deliverables. We obtained peptides of endogenous origin from Ii^- breast cancer vaccines, and peptides of exogenous origin from Ii^+ vaccines (completed).

Task 2. Determine if the peptides identified in Task 1 are efficacious for activating type T-cells from breast cancer patients (completed).

Task 2a. Determine if the candidate peptides identified in Objective 1 activate CD4⁺ T cells from healthy donors and from breast cancer patients that react with wild type MHC II⁺ breast cancer cells (completed)

Task 2b. Determine if the MHC II-restricted candidate peptides identified in Objective 1 are expressed by breast cancer cells (in progress).

Task 2c. Determine if MHC II tumor peptides obtained from Ii⁻ vaccine cells are different from peptides obtained from Ii⁺ cells (in progress).

Task 2d. Determine if the peptides activate CD4⁺ T-cells in the presence of immune suppression.

Outcomes/Products/Deliverables. This task will identify the subset of the peptides identified in Task 1 from Ii⁻ vaccine cells that efficiently activate tumor-reactive CD4⁺ T-cells from breast cancer patients, even in the presence of immune suppression. (in progress)

Task 3. Determine if CD4⁺ T-cells activated with the candidate peptides facilitate a more robust CD8⁺ T-cell response by breast cancer patients. (in progress)

Outcomes/Products/Deliverables. This task will identify the subset of the peptides that efficiently activate type 1 tumor-reactive CD4⁺ T-cells and facilitate the most robust CD8⁺ T-cell response by breast cancer patients. These peptides are likely candidates for use in future breast cancer vaccines.

Reportable outcomes

Presentations

1. Olesya Chornoguz, Lydia Grmai, Alexei Gapeev, Michael O'Neill, Suzanne Ostrand-Rosenberg. "MHC II peptides as breast cancer immunotherapy agents". 59th ASMS Conference on Mass Spectrometry, Denver, CO, June 5 – June 9, 2011 (poster)
2. Olesya Chornoguz, Lydia Grmai, Alexei Gapeev, Michael O'Neill, Suzanne Ostrand-Rosenberg. "MHC II-restricted peptides derived from invariant chain negative breast cancer cell-based vaccines activate healthy donor T-cells". American Association of Immunologists 97th Annual meeting, San-Francisco, CA May 13-17, 2011 (oral presentation).
3. Olesya Chornoguz, Lydia Grmai, Alexei Gapeev, Michael O'Neill, Suzanne Ostrand-Rosenberg. "MHC II-restricted peptides derived from invariant chain negative breast cancer cell-based vaccines activate healthy donor T-cells". Era of Hope, DOD sponsored conference, August 2 – 5, 2011, Orlando, FL
4. Olesya Chornoguz¹; Lydia Grmai¹; Pratima Sinha¹; Konstantin Artemenko³; Roman Zubarev²; Suzanne Ostrand-Rosenberg¹. "Inflammation-induced Myeloid-Derived Suppressor Cells have enhanced resistance to apoptosis." American Association of Immunologists 97th Annual meeting, Baltimore, MD. May 7-11, 2010 (oral presentation).
5. Olesya Chornoguz¹; Lydia Grmai¹; Pratima Sinha¹; Konstantin Artemenko³; Roman Zubarev²; Suzanne Ostrand-Rosenberg¹. "Pathway Analysis reveals apoptosis as a regulator of breast cancer induced Myeloid-Derived Suppressor Cells." 58th ASMS Conference on Mass Spectrometry, Salt Lake City, UT, May 23 – 27, 2010 (oral presentation).
6. Olesya Chornoguz, Minu K. Srivastava, Suzanne Ostrand-Rosenberg, "Mass Spectrometry Analysis of the MHC Class II peptide repertoire of cell-based breast cancer vaccines transduced with the class II Transactivator in the presence or absence of Invariant Chain" American Association for Cancer Research Annual Meeting, San-Diego, CA, April 12th – 16th 2008 (invited oral presentation).

Publications

1. Chornoguz O, Grmai L, Sinha P, Artemenko KA, Zubarev RA, Ostrand-Rosenberg S. "Proteomic pathway analysis reveals inflammation increases myeloid-derived suppressor cell resistance to apoptosis." Mol Cell Proteomics. 2011 Mar;10(3):M110.002980. Epub 2010 Dec 29.
2. Sinha P, Chornoguz O, Clements VK, Artemenko KA, Zubarev RA, Ostrand-Rosenberg S. Myeloid-derived suppressor cells express the death receptor Fas and apoptose in response to T cell-expressed FasL. Blood. 2011 Mar 30. [Epub ahead of print]

Awards

- American Association of Immunologists abstract Trainee travel Award for attendance of American Association of Immunologists 98th Annual meeting, San-Francisco, MD. May 13-17, 2011, May 2011

Informatics

We are in the process of publishing the MHC II peptide data that was collected in Aim 1. As a result of this published work there will be a published database of breast cancer cells-derived MHC II peptide repertoire both in the absence and the presence of Invariant Chain. This database will facilitate development of future breast cancer peptide vaccines.

Conclusions

- Task 1 of Research plan was successfully completed (outlined in Statement of Work, SOW). MHC II peptides presented by Ii⁺ and Ii⁻ MHC II breast cancer vaccines were identified.
- It has been demonstrated that Ii⁺ and Ii⁻ vaccines present distinct, but overlapping peptide repertoire
- It has also been showed that Invariant chain influences MHC II peptide repertoire
- Seven peptides predicted to have the highest affinity to MHC II molecules were chosen for in vitro CD4⁺ T-cell activation and all of them have a comparable or better immunogenicity as a well-characterized breast cancer peptide Her2.
- I was able to show that peptide 48 activates breast cancer patient's PBMC.
- Most relevant specific tasks outlined in Training Plan and Milestones and Deliverables sections of SOW were completed.
- More specifically I have presented two posters and gave one oral presentations at several national meetings during the second year of training period. (See reportable outcomes).
- I have also received a travel award to present my research at American Association of Immunology conference. (See reportable outcomes).
- My first first-author manuscript titled "Proteomic Pathway Analysis Reveals Inflammation Increases Myeloid-Derived Suppressor Cell Resistance to Apoptosis" has been published in Molecular and Cellular Proteomics journal. (See reportable outcomes).

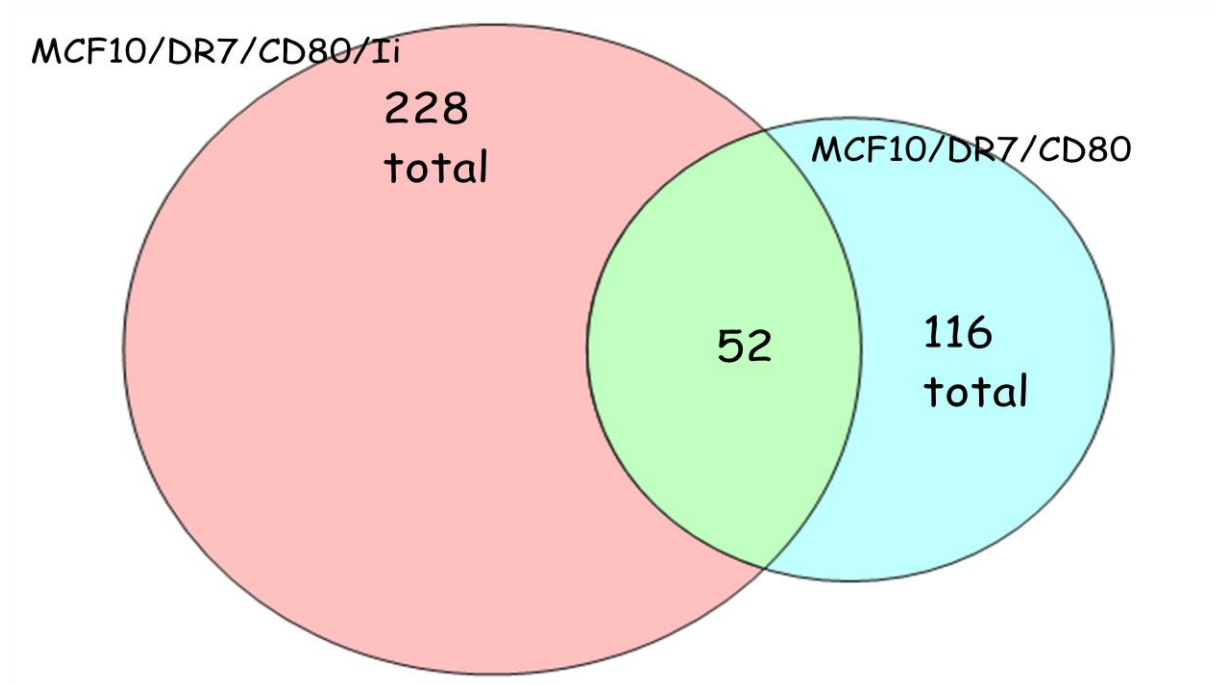
- I was also a co-author on the following manuscript: “Myeloid-derived suppressor cells express the death receptor Fas and apoptose in response to T cell-expressed FasL”. This manuscript was published in Blood journal, in May of this year. (See reportable outcomes)
- Manuscript describing all work done on the MHC II peptide project is in preparation currently.

References

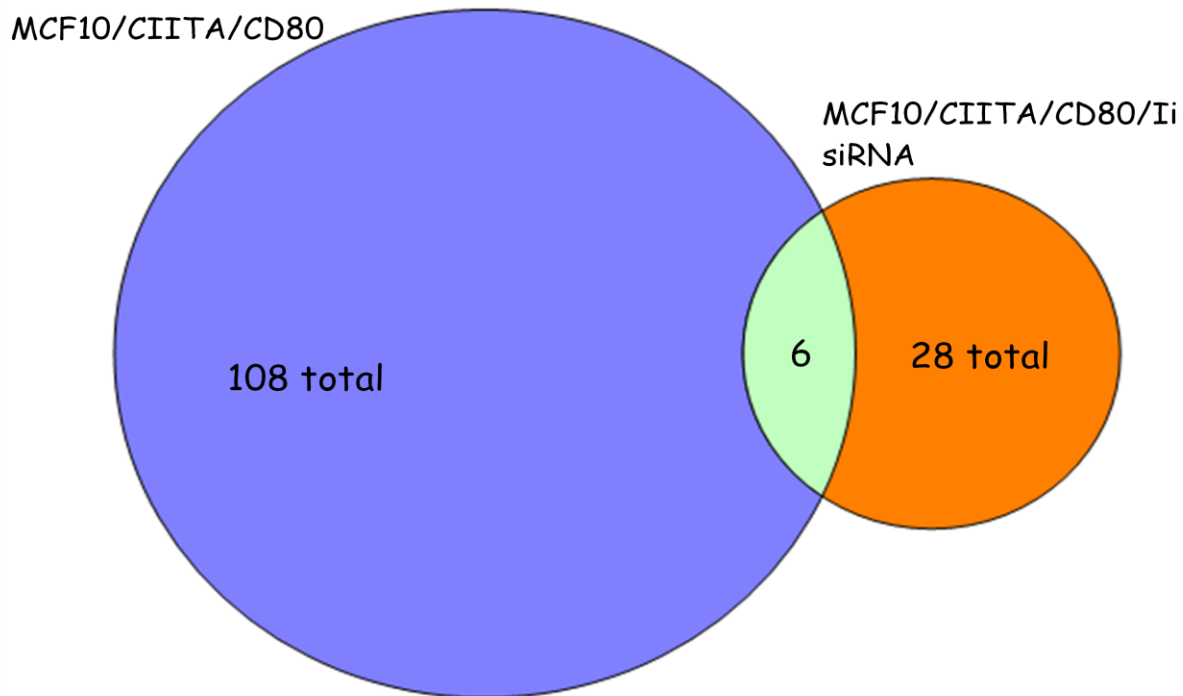
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7. Thompson, J.A., et al., *The absence of invariant chain in MHC II cancer vaccines enhances the activation of tumor-reactive type 1 CD4+ T lymphocytes*. Cancer Immunol Immunother, 2008. 57(3): p. 389-98.
8. Srivastava, M.K., et al., *Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells*. Cancer Immunol Immunother, 2008. 57(10): p. 1493-504.
9. Sotiriadou, R., et al., *Peptide HER2(776-788) represents a naturally processed broad MHC class II-restricted T cell epitope*. Br J Cancer, 2001. 85(10): p. 1527-34.

Fig 1. MHC II peptides identified from 4 vaccines. These peptides were identified as a result of 2 MHC II peptide purifications. Peptides from each purification were analyzed twice on LTQ mass spectrometer; only peptides common between the two purifications were considered as reliable identifications.

A)



B)



Sequence and SOR#	Protein	Vaccine	Sub cellular localization
RQTVAVGVKAVDKKA, SOR-42	Translation elongation factor, EEF1A1 protein	MCF10/CIITA/CD80/Ii siRNA, present in Ii + as well	Nucleus, nucleolus, cytoplasm
KVAPAPAVVKKQEAKK, SOR-43	60S ribosomal protein L7a	MCF10/CIITA/CD80/Ii siRNA	<u>Predicted:</u> Mitochondrion and cytoplasm
EEEALANASDAELCDIAAILGMHTLM, SOR-44	Tropomodulin 1	MCF10/DR7/CD80, present in Ii+ as well	<u>Predicted:</u> Nucleus and cytoplasm
SESAAAPAFASSSSEVNPAPKFHW, SOR-46	Hypothetical protein, isoform 1	Both	Protein not present in the database
RTYAGGTASATKVSASSGATSKS, SOR-45	Calpastatin, isoform e, calcium-dependent cysteine protease) inhibitor	Both	Protein not present in the database
GKFYLVIEELSQLFRSLVPIQL, SOR-48	Hypothetical protein	MCF10/CIITA/CD80/Ii siRNA	Protein not present in the database
RRMRLTHCGLQEKHL, SOR-47	NLRC5 protein, IFN-specific response element	MCF10/DR7/CD80	<u>Predicted:</u> Cytoplasm, nucleus

Table 1. Seven peptides that were derived from Ii⁻ vaccines and were predicted to have the highest binding affinity to HLA-DR7 MHC II, using Artificial Neural Networks (ANN). The first column shows peptide sequence and a short-hand notation (SOR-xx number). Second column shows the protein from which the peptide was derived. Some of the peptide (SOR-46 and SOR-48) are derived from peptides of unknown function, thus they are named as hypothetical proteins in the FASTA database. The third column shows which vaccine or vaccines the peptide was derived from. The fourth column shows predicted or published sub cellular localization of the proteins from which the peptides were derived from. LOCATE web-tool (<http://locate.imb.uq.edu.au>) was used to derive predicted or published sub cellular localization data. Three of the proteins of interest were not present in LOCATE database, and therefore are indicated as “Protein not present in the database”. Peptides highlighted in blue have already been tested for their ability to activate CD4⁺ T-cells as measured by IFN γ secretion

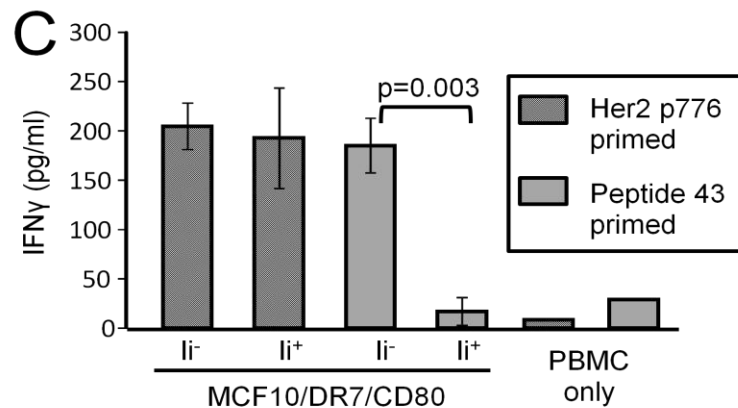
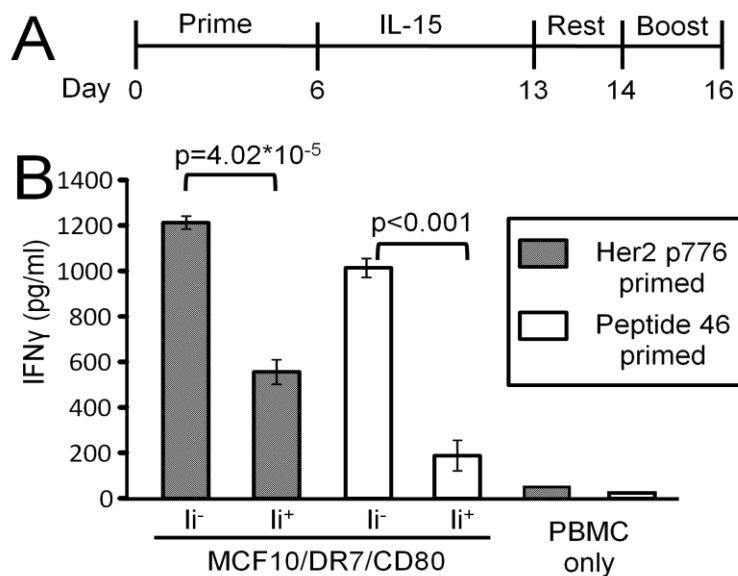
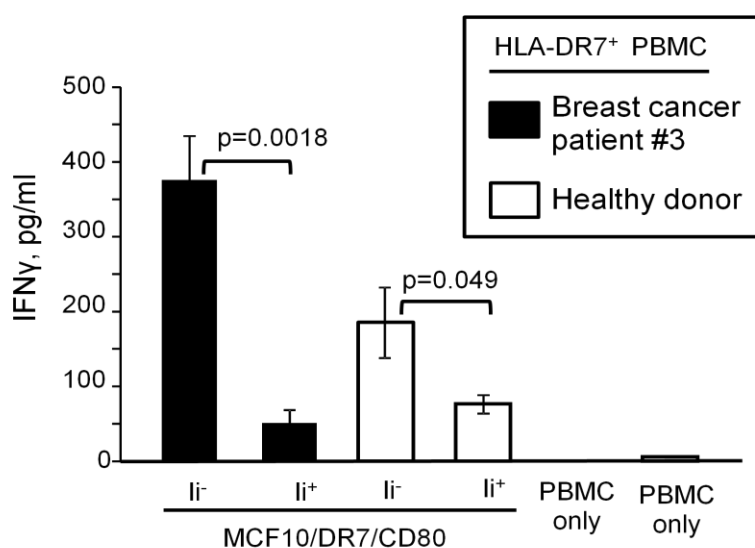


Figure 2. HLA-DR7-restricted peptides derived from DR⁺Ii⁻ MCF10⁻ vaccine cells are better activators of tumor-specific T-cells than peptides from DR⁺Ii⁺ MCF10 transfectants. (A) T cell activation scheme: HLA-DR7⁺ PBMC were primed with peptide for 6 days, expanded with IL-15 for 7 days, rested for 24 hours, and IFN γ production was measured by ELISA after a 48 hour boost with DR7⁺/CD80⁺/Ii⁺ or DR7⁺/CD80⁺/Ii⁻ cells. HLA-DR7⁺ healthy donor PBMC were primed with Her2 p776 and either Peptide 46 (B) or Peptide 43 (C), and boosted with the indicated vaccine cells or MCF10 transfectants.

Figure 3. Peptide 48 derived from DR⁺/CD80⁺/Ii⁻ MCF10 vaccine cells activates T-cells from breast cancer patients. PBMC from an HLA-DR7⁺ healthy donor or from breast cancer patient #3 were primed with Peptide 48 (GKFYLVIEELS QLFRSLVPIQL) and boosted with the indicated vaccine cells or MCF10 transfectants. T-cell activation was quantified by measuring IFN γ production.



Peptide Sequence , ID#, and cell source	CD4 ⁺ T-cell activation relative to Her2 p776
RQTVAVGVKAVDKKA, 42 (Ii ⁺ , Ii ⁻)	96%
KVAPAPAVVKKQEAKK, 43 (Ii ⁻)	90%
EEEALANASDAELCDIAAILGMHTL, 44 (Ii ⁻ , Ii ⁺)	107%
RTYAGGTASATKVSASSGATSKS, 45 (Ii ⁻)	333%*
SESAAAPAFASSSSEVNPAPKFHW, 46 (Ii ⁻)	83%*
RRMRLTHCGLQEKHL, 47 (Ii ⁻)	52%
GKFYLVIEELSQLFRSLVPIQL, 48 (Ii ⁻)	120%

Table 2. The 7 peptides with predicted highest binding affinity to HLA-DR7 efficiently activated CD4⁺ T-cells from HLA-DR7⁺ healthy donors. Summary of 12 T-cell activation experiments with 7 different peptides. Response >100% indicates priming with the indicated peptide yielded more IFN γ than priming with Her2 p776. Asterisk indicates that the peptide yielded significantly more (peptide 45) or less (peptide 46) IFN γ upon priming with these corresponding peptides as compared with Her2 p776 peptide.